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THE STAINING OF SPIROCHETES IN COVER-GLASS SMEARS BY THE SILVER-AGAR METHOD*

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The methods in practical use at the present time for the immediate clinical and laboratory demonstration of spirochetes are the dark-field and the India-ink methods. Both of these methods are dangerous for the inexperienced laboratory worker of the type common today in the smaller hospitals scattered throughout the country. Within the last year we have been consulted with reference to three cases of carcinoma of the lip and mouth and one of the genitalia in which mouth and smegma spirochetes were mistaken in the dark-field for Spirochaeta pallida. In one of these cases the patient was treated for syphilis until hopeless generalized metastases developed, and in this case the mistaken diagnosis was made by an expert in the use of the dark-field and the clinical recognition of syphilis. We have reason to believe that such mistakes are not uncommon. Certain forms of spirochetes of the mouth so approach the pallida in appearance in the dark field that morphologic differentiation by this method is sometimes impossible. We believe that fine morphologic differences are more easily recognizable in the stained smear, and regard the use of the latter as the safest procedure in the clinical diagnosis of syphilis. There has, however, not yet been devised any simple staining method for the demonstration of spirochetes in cover-glass smears. No stain or dye has yet been discovered that will stain spirochetes sufficiently well for diagnostic purposes. The silver-impregnation methods are the only ones that will. We offer here a method of application of silver-impregnation to the study of spirochetes in smears.

Method for Silver-Impregnation of Spirochetes in Smears on Cover-Glasses

1. Prepare smears on No. 1 cover-glasses.
2. Dry thoroughly in the air.
3. Place in absolute alcohol 3 - 5 minutes.
4. Wash in distilled water.

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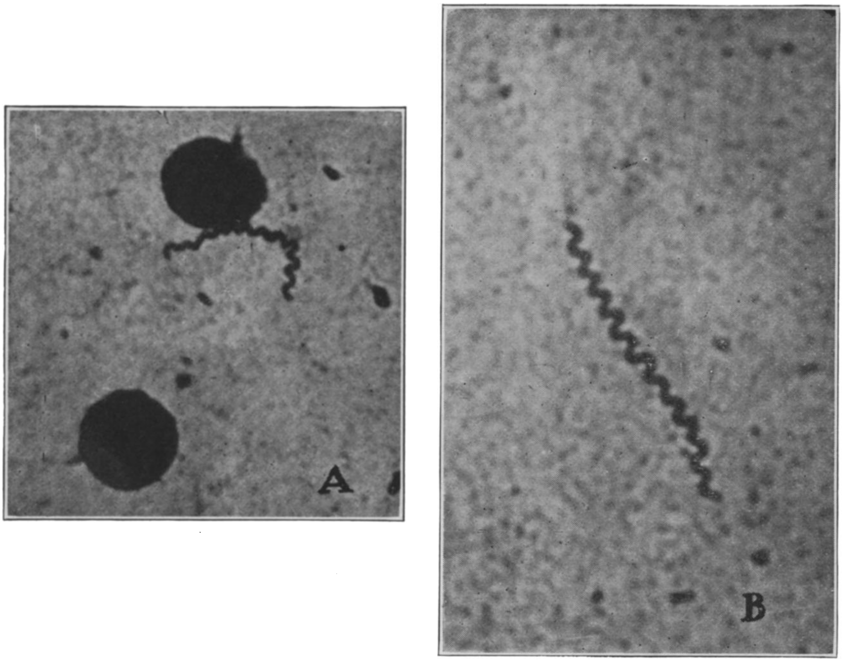

Fig. 1.—Smear from chancre, stained by Warthin-Starry silver-agar method. A, Spirochaeta pallida with two red blood cells, photomicrograph, B. & L., oil-immersion 2 mm., compensating ocular No. 4, bellows length 85 cm. B, another organism from same smear with bellows length of 155 cm.

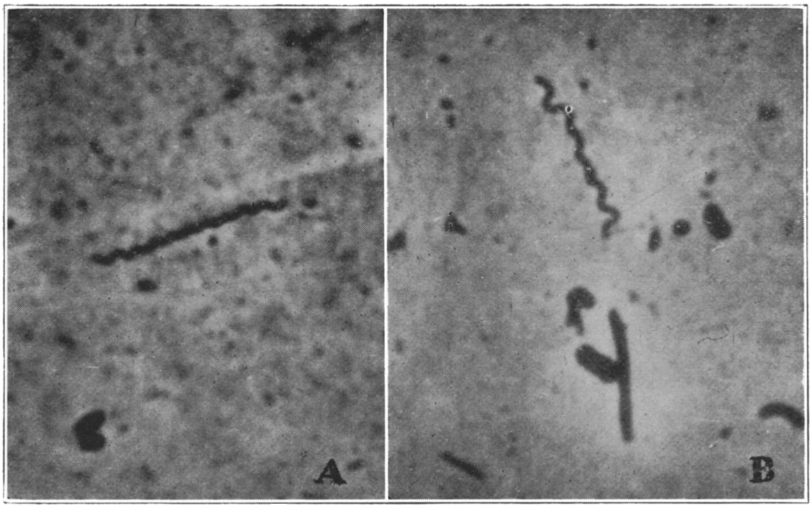

Fig. 2.—Smear from gums. A and B, two types of mouth spirochetes, the ones most commonly mistaken in the dark-field for pallida. Photomicrograph, B. & L., oil-immersion 2 mm., compensatory ocular No. 4, bellows length 155 cm. Smear stained by Warthin-Starry silver-agar method.

(If hydrogen peroxide is used to clear background, the smear is placed in concentrated hydrogen peroxide for 5 - 20 minutes, and then washed thoroughly in distilled water.)

5. Rinse cover-glass with smear in 2% silver nitrate. Cover the smear side with another perfectly clean cover-glass also rinsed in the silver nitrate solution. Place the adherent pair of cover-glasses carefully, so as not to separate them, in a bottle of 2% silver nitrate, and place in an incubator for 1 -2 hours; then remove the cover-glasses from the silver nitrate solution and separate them.

6. Place the cover-glass with the smeared side up in the following mixture:

2% silver nitrate solution	3 c c
Warm 10% aqueous gelatin solution	5 c c
Warm glycerol	5 c c
Warm 1.5% agar suspension	5 c c
5% aqueous hydroquinone solution	2 c c

7. After the solution is reduced remove and rinse in 5% sodium thiosulphate solution.

8. Rinse in distilled water.

9. Absolute alcohol, xylol, balsam.

Discussion of the Method.—Clean cover-glasses are essential. The cover-glass smears are prepared in the usual way. It is important that they be thoroughly dried in the air before attempting to stain them. Long exposure to air does not affect the staining quality if they are protected from dust and dirt. Dried smears containing Leptospira icteroides stained readily after standing 4 weeks. After drying, the smears are placed in absolute alcohol for 3-5 minutes, and are then washed with 2 changes of distilled water. It is essential that no other method of fixation than drying in air followed by absolute alcohol be used.

The silver-impregnation is carried out in wide-mouthed dark bottles fitted with tightly fitting corks. The smear is taken from the distilled water, and rinsed in 2% silver nitrate solution; the smeared side is then covered with another perfectly clean cover-glass also rinsed in the silver nitrate solution, so that the wet cover-glasses adhere. The silver nitrate solution should be fresh, not over 6 - 7 days old. During this time the silver nitrate solution can be used over and over if kept in the dark bottle and free from contamination. The adhering pair of cover-glasses are put into the bottle so that they stand on edge against the side of the bottle, and enough silver nitrate is poured into the bottle to come about half way up the cover-glasses. If round bottles are used a small meniscus is formed between the cover-glasses and the side of the bottle, thus holding them in place. It is a good routine measure always to place the smeared cover-glass next to the wall of the bottle, so that there will be no danger of getting the cover-glasses mixed and placing the wrong one in the reducing mixture.

The reducing mixture is prepared by mixing the silver nitrate solution, gelatin and glycerol in order. This mixture is thoroughly stirred, and the agar suspension stirred in last. The hydroquinone solution is added just before using. About 2 c c give the best results. If the reduction takes place too rapidly add less, if too slowly, add more. The agar suspension should be carefully made as follows: One and a half gm. of agar are broken up fine, and placed in 20 - 30 c c of distilled water, and allowed to soak for a few minutes until the agar is saturated with water. The excess is then poured off and the agar washed with

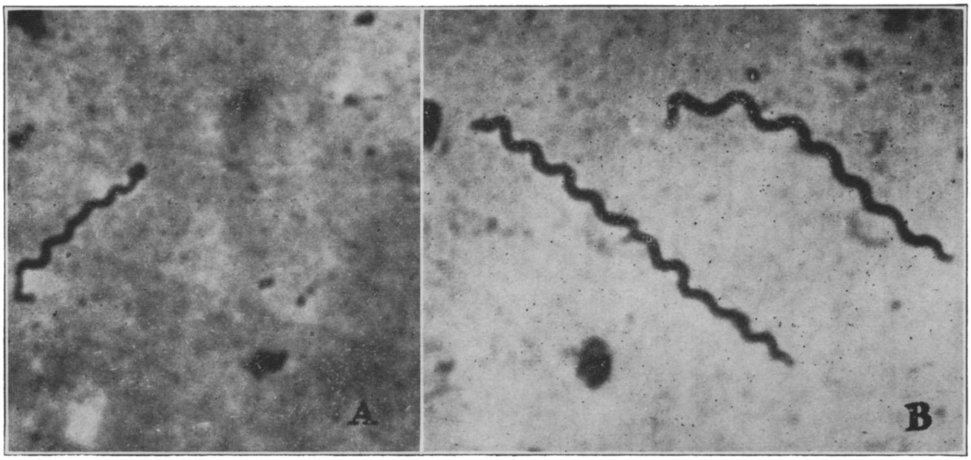

Fig. 3.—Smear of rat's blood, stained by Warthin-Starry hydrogen-peroxide, silver-agar method. A, Sp. obermeieri. Photomicrograph, B. & L., oil-immersion 2 mm., compensatory ocular No 4, bellows length 85 cm. B, two Sp. obermeieri from same smear, same stain, with bellows length of 155 cm

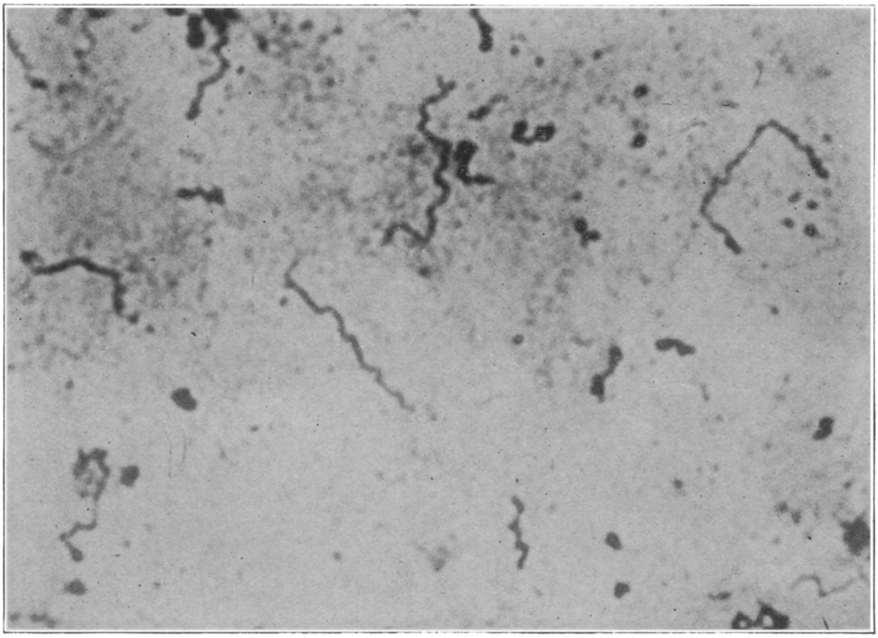

Fig. 4.—Smear from old culture of Sp. icterohaemorrhagica, stained by Warthin-Starry hydrogen-peroxide, silver-agar method. Photomicrograph, B. & L., oil-immersion 2 mm., compensatory ocular No. 4, bellows length 85 cm. Organisms show some involution.

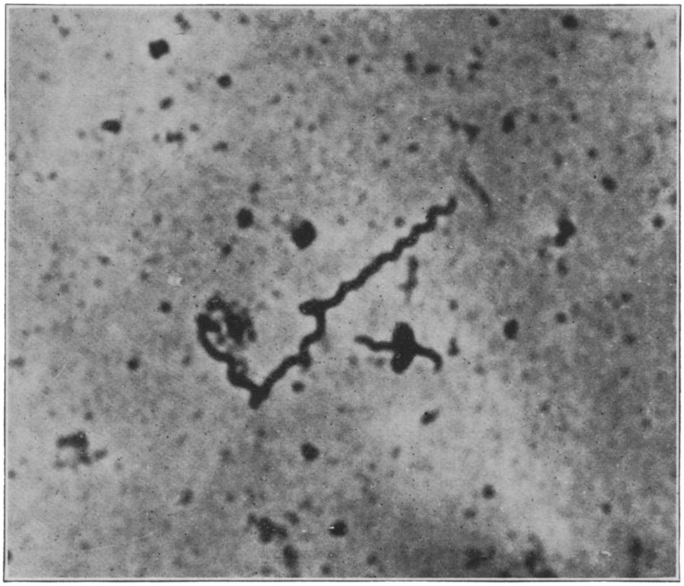

Fig. 5.—Spirochaeta icterohaemorrhagica from same stained smear as preceding, taken with a bellows length of 155 cm.

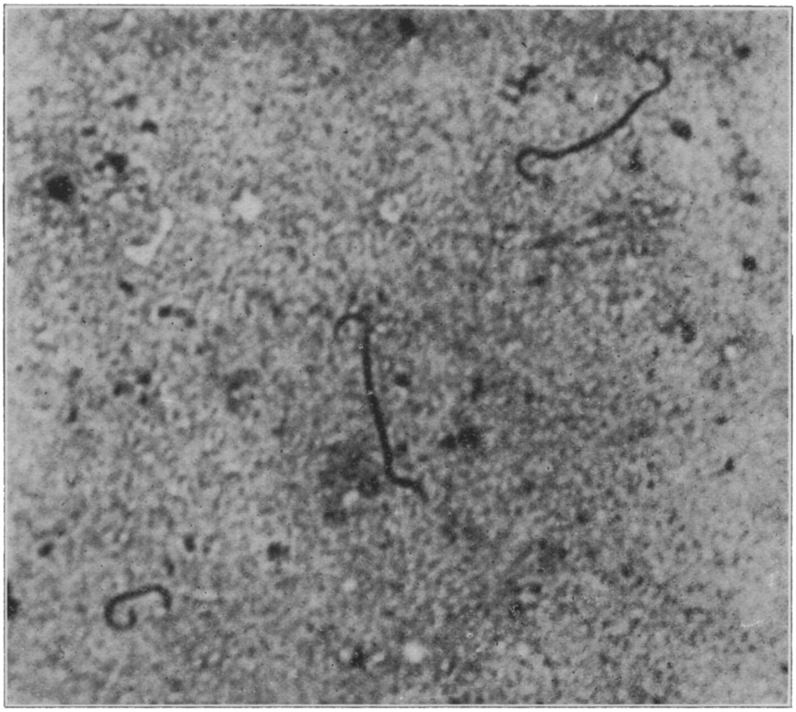

Fig. 6.—Leptospira icteroides in smear from guinea-pig's liver, stained with Warthin-Starry hydrogen-peroxide silver-agar method. Photomicrograph, B. & L., oil-immersion 2 mm., compensatory ocular No. 4, bellows length 100 cm. Organisms show typical "shepherd's crook ends." "Granule" near motile segment also indicated.

several changes of distilled water; 100 c c of distilled water is then added, and it is carefully brought to the boiling point with constant stirring. When the agar has gone into fine suspension the mixture is poured into a clean bottle, corked and allowed to cool. As the agar thickens it is occasionally shaken, and when it begins to set it is thoroughly broken up by violent shaking. It is then placed on top of the paraffin oven, after which it will remain a thick heavy mixture just fluid enough barely to run. All of the solutions used should be made up in clean porcelain or glass ware, and not in metal containers.

The hydroquinone is added just before using. After it has been added the mixture is stirred vigorously for a short time, when it is poured into staining dishes, and the smears removed from the silver nitrate solution, the cover-glasses separated, and the one having the smear is immersed in the reducing mixture for a number of seconds (30 seconds to 2 minutes) until the reduction is complete. The smears turn a light brown; if very thin they may scarcely change color. When reduced, the smears are removed and placed in a 5% sodium thiosulphate solution for a few seconds. They are then rinsed in water, dehydrated in absolute alcohol, cleared in xylol and mounted in balsam. The longer the smears are in the reducing fluid the darker they are; it is, therefore, advisable to leave them in it until the reduction is nearly or wholly completed.

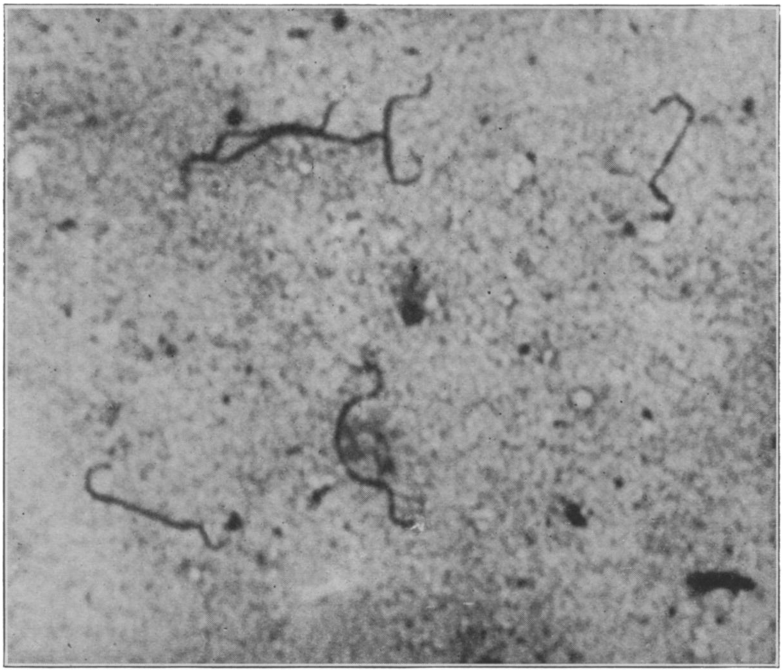

Fig. 7.—Leptospira icteroides in smear from macerated guinea-pig's liver, stained by Warthin-Starry hydrogen peroxide, silver-agar method. Photomicrograph with B. & L. oil-immersion 2 mm., compensatory ocular No. 4, bellows length 100 cm.

The use of the extra cover-glass in the silver solution seems to be essential. Good results are rarely obtained if the smear is left uncovered, as the serum and cellular elements of the smear take a deep brown stain and the spirochetes are usually indistinguishable.

It is essential that all reagents used be chemically pure and free from all contamination. The alcohol must be pure; if it contains phenol, mercuric chlorides, etc., the smears are easily mordanted, and the staining of the spirochetes becomes difficult.

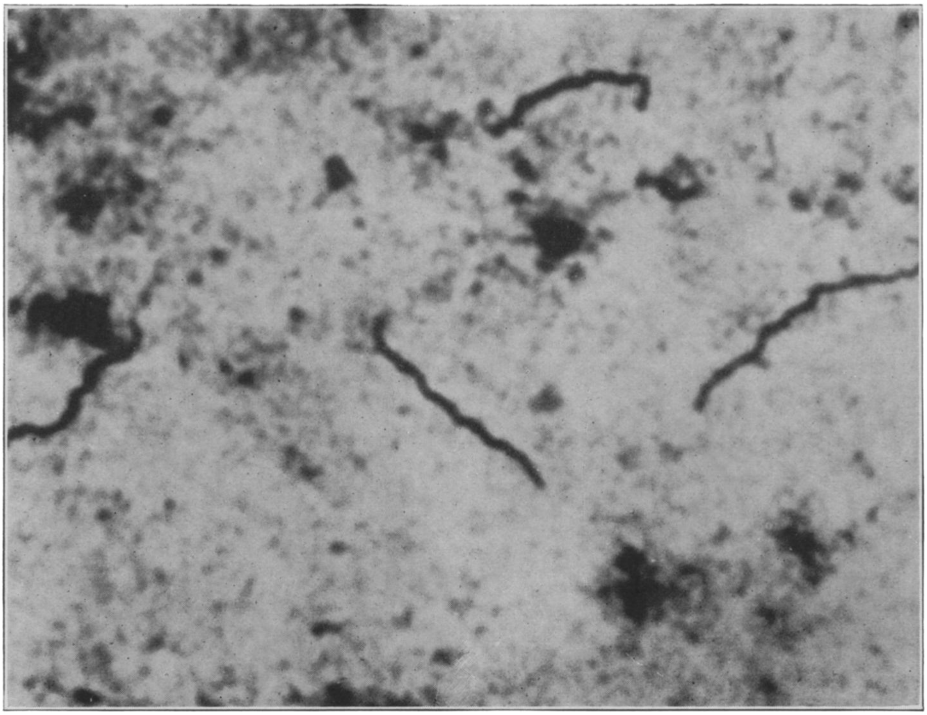

Fig. 8.—Leptospira icteroides in smear of macerated guinea-pig's liver. Same preparation. Taken with bellows length of 155 cm.

The spirochetes in the stained smears should appear black against a light background. In heavy smears with much serum or cell material we have found it of great advantage to use hydrogen peroxide to clear up the background. Between steps 4 and 5 of the method the cover-glass is placed in concentrated hydrogen peroxide for 5 - 20 minutes. It is then washed in 2 changes of distilled water before proceeding with the silver-impregnation in No. 5. In smears made from macerated liver we have found it possible to secure nearly colorless backgrounds for the spirochetes. The hydrogen peroxide must be pure and free from such impurities as barium chloride and other substances commonly found in commercial hydrogen peroxide. We have found that put up in pure form in slightly acidulated water, made by Parke, Davis & Co., to be satisfactory. With care the same solution can be used over and over for several weeks.

The spirochetes are increased in apparent size by this method, particularly in so far as their thickness is concerned. The precipitation of silver is chiefly a surface reaction on the spirochete. There is apparently some difference between different forms of spirochetes with relation to the intensity of this precipitation. The spirochetes of syphilis, relapsing fever, mouth and smegma precipitate silver much more intensely than Spirochaeta icterohaemorrhagica and Leptospira icteroides, and the thickness of the first-named group is increased proportionately, that of Sp. obermeieri about three times. This magnification of the size of the organisms is of the greatest advantage in diagnostic work, as every morphologic detail of the organism is proportionately increased in size and intensity and differential morphological characteristics are accentuated. The number, size and character of the turns of the organisms are easily determined, more so than by any other method.

The stained smears are not always permanent. After 4-6 weeks some of them fade, while others have retained their original intensity for a year and longer. We have found this fading particularly marked in the case of Leptospira icteroides. When for purposes of record it is desirable to make permanent preparations we have found that the smears made according to our method can be stabilized and, so far as our present experience goes, made permanent by toning with gold chloride. We have found Perrin's[1] method very satisfactory. After the smears are stained they are washed in a solution of sodium hyposulphite, then with distilled water and then toned in the following solution:

Ammonium sulphocyanide	6.25 gm.
Tartaric or citric acid	.50 gm.
Sodium chloride	1.25 gm.
Distilled water	250 c c
Solution of gold chloride (1 : 100)	6.25 c c

After a short time in this solution (5-15 minutes) the stain turns to a blue-black color. The smears are then washed in distilled water, dehydrated and cleared, and mounted in balsam.

We believe that our method of demonstrating spirochetes in cover-glass smears by silver-agar silver impregnation is the surest and safest diagnostic method yet devised. It should replace the use of the dark-field and the India-ink method in the diagnosis of syphilis. The morphologic details of Spirochaeta pallida and the mouth and smegma organisms so often mistaken for it by workers using the dark-field or India-ink method are so accentuated that differential diagnosis is made much easier. The India-ink method is especially dangerous for inexperienced workers. If spirochetes are present in the smear they will be stained by our method if directions are followed. The method is not formidable as it may seem because of the full directions given. It is easily acquired by laboratory workers. Aside from the time required for silver-impregnation in the incubator, it is a relatively short method, and a large number of smears can be kept going in different bottles at the same time. For bacteriologists and laboratory workers engaged in spirochete studies this method is of the greatest advantage in recovering organisms from the organs of inoculated animals, or from the blood or urine. It lends itself particularly well to the laboratory study of Sp. icterohaemorrhagica and Leptospira icteroides. Nevertheless, it is in its application to the clinical diagnosis of syphilis that it possesses its greatest value.

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[1] Arch. f. Dermat. u. Syphilis, 1920, 21, p. 354.